ANALYTIC VALIDITY

Question 8: Is the test qualitative or quantitative?
Question 9: How often is a test positive when a mutation is present (analytic sensitivity)?
Question 10: How often is the test negative when a mutation is not present (analytic sensitivity)?
Question 11: Is an internal quality control program defined and externally monitored?
Question 12: Have repeated measurements been made on specimens?
Question 13. What is the within- and between-laboratory precision?
Question 14: If appropriate, how is confirmatory testing performed to resolve false positives in a timely manner?
Question 15: What range of patient specimens have been tested?
Question 16: How often does the test fail to give a useable result?
Question 17: How similar are results obtained in multiple laboratories using the same, or different, technology?

ANALYTIC VALIDITY

Question 8. Is *BRCA1/2* mutation testing qualitative or quantitative?

Summary

- Testing for *BRCA1/2* mutations is qualitative
- There are three possible categories of results for full DNA sequencing:
  - positive for a deleterious mutation
  - negative for a deleterious mutation
  - genetic variant (three types – suspected deleterious, favor polymorphism, and uncertain clinical significance).

Testing for *BRCA1/2* mutations is qualitative. There are three possible categories of results for full DNA sequencing: 1) positive for deleterious mutation, 2) negative for deleterious mutation, and 3) genetic variant (three types – suspected deleterious, favor polymorphism and uncertain clinical significance). Testing targeted at specific mutations (e.g., a mutation identified in an index case or the three mutations common in Ashkenazi Jewish women) will yield only positive or negative results; all of the mutations being tested are known to have clinical significance. Myriad Genetic Laboratories (Myriad) further breaks down these results into categories as follows: (www.myriadtests.com/provider/doc/tech_specs_brac.pdf, under Technical Specifications).

"Positive for a deleterious mutation": Includes all mutations (nonsense, insertions, deletions) that prematurely terminate (truncate) the protein product of *BRCA1* at least 10 amino acids from the C-terminus, or the protein product of *BRCA2* at least 110 amino acids from the C-terminus (based on documentation of deleterious mutations in *BRCA1* and *BRCA2*). In addition, specific missense mutations and non-coding intervening sequence (IVS) mutations are recognized as deleterious on the basis of data derived from linkage analysis of high risk families, functional assays, biochemical evidence and/or demonstration of abnormal messenger ribonucleic acid (mRNA) transcript processing.

"Genetic variant, suspected deleterious": Includes genetic variants for which the available evidence indicates likelihood, but not proof, that the mutation is deleterious. The specific evidence supporting such an interpretation will be summarized for individual variants on each such report.

"Genetic variant, favor polymorphism": Includes genetic variants for which available evidence indicates that the variant is highly unlikely to contribute substantially to cancer risk. The specific evidence supporting such an interpretation will be summarized for individual variants on each such report.

"Genetic variant of uncertain significance": Includes all missense mutations and mutations that occur in analyzed intronic regions whose clinical significance has not yet been determined, as well as chain-terminating mutations that truncate *BRCA1* and *BRCA2* distal to amino acid positions 1853 and 3308, respectively.

"No deleterious mutation detected": Includes non-truncating genetic variants observed at a frequency of approximately 2 percent of a suitable control population (providing that no data suggest clinical significance), as will as all genetic variants for which published data demonstrate absence of substantial clinical significance. Also includes mutations in the protein-coding region that neither alter the amino acid sequence nor are predicted to significantly affect exon splicing, and base pair alterations in non-coding portions of the gene that have been demonstrated to have no deleterious effect on the length or stability of the mRNA transcript. Data on polymorphic variants are available upon request. There may be uncommon genetic abnormalities in *BRCA1* and *BRCA2* that will not be detected by BRACAnalysis™ (see last paragraph of this question). This analysis, however, is believed to rule out the majority of abnormalities in these genes, which are believed to be responsible for most hereditary susceptibility to breast and ovarian cancer.

"Specific variant/mutation not identified": Specific and designated deleterious mutations or variants of uncertain clinical significance are not present in the individual being tested. If one (or rarely two) specific deleterious mutations have been identified in a family member, a negative analysis for the specific mutation(s) indicates that the tested individual is at the general population risk of developing breast or ovarian cancer.

Change of interpretation and issuance of amended reports: If and whenever there is a change in the clinical interpretation of a specific reported variant, an amended test report will automatically be provided by Myriad Genetic Laboratories.

Limitations of DNA Sequencing

- DNA sequencing is able to detect only point and small mutations
- Promoter regions are not analyzed
- Large genomic rearrangements and some types of errors in RNA transcript processing are not detected by the usual polymerase chain reaction (PCR)-based methodologies, including Myriad's sequencing technique. These defects represent an estimated 10 to 15 percent of all disease-causing mutations in the general population (Puget *et al.*, 1999; Unger *et al.*, 2000) and up to 36 percent in the Dutch population. (Petrij-Bosch *et al.*, 1997).
- There may be limited portions of either *BRCA1* or *BRCA2* for which sequence determination can be performed only in the forward or reverse direction
- Unequal allele amplification may result from rare polymorphisms under primer sites

Question 18 examines the issue of clinical validity in more detail. It has been estimated that between 63 and 67 percent of expected deleterious mutations showing linkage to *BRCA1* are identified by PCR-based mutation-detection assays. (Ford *et al.*, 1998; Gayther and Ponder, 1997) In August of 2002, Myriad added a panel to its comprehensive analysis that identifies five deleterious large recurrent rearrangements in the *BRCA1* gene. This panel detects four large deletions in exons 8 and 9, exon 13, exon 22, and exons 14-20, and one duplication in exon 13.

ANALYTIC VALIDITY

Question 9. How often is the test positive when a mutation is present (sensitivity)?
Question 10. How often is the test negative when a mutation is not present (specificity)?

Summary

- External proficiency testing schemes are the only major reliable source currently available for computing analytic sensitivity and specificity. The following caveats should be kept in mind, however, when examining these estimates. First, external proficiency testing schemes are designed to be educational. It is also likely that both research and clinical laboratories participate. In spite of these shortcomings, this source of data can be useful in establishing a baseline of performance.
- Due to patent restrictions, full gene sequencing for clinical purposes can only be done by Myriad Genetic Laboratories. Thus, the most relevant information about analytic performance needs to be specific to that laboratory. However:
  - Myriad Genetic Laboratories provides only a summary statement of analytic sensitivity and specificity, with no supporting data available for external review
  - The external proficiency survey available in the U.S. is of limited relevance, because the survey is restricted to three mutations, and many participants do not use sequencing to identify mutations
  - External proficiency testing available in Europe is also of limited relevance, because the survey specifies which exons to examine and assesses only analytic sensitivity. A variety of methodologies is utilized.
- Based on data from the European Molecular Genetics Quality Network
  - The overall error rate is 2.7 percent (95 percent CI 1.6 to 4.2%)
  - Analytic sensitivity is 97.1 percent (95 percent CI 95.2 to 98.5%)
- Based on data from the American College of Medical Genetics and the College of American Pathologists Molecular Genetics Laboratory Survey
  - The overall error rate is 0.0 percent (95 percent CI 0.0 to 4.6%)
  - The analytic sensitivity is 100 percent (95 percent CI 93.0 to 100%)
  - The analytic specificity is 100 percent (95 percent CI 96.0 to 100%)

Definitions

Analytic performance is summarized by the sensitivity and specificity of the detection system.

Analytic sensitivity is the proportion of positive test results, when a detectable mutation is present (i.e., the test is designed to detect that specific mutation). The analytic sensitivity may also be called the analytic detection rate. Another way of expressing analytic sensitivity would be to divide the true positives by the sum of the true positives and false negatives. False negative results could be due to technical errors in the analytic phase (e.g., sample placement, contamination, expired reagents and cross-reactivity) or to administrative/clerical errors in the pre-analytic or post-analytic phases (e.g., incorrect interpretation of correct analytic result, sample mislabeling and incorrectly copying a correct result).

Analytic specificity is the proportion of negative test results when no detectable mutation is present. Another way of expressing analytic specificity would be to divide the true negatives

by the sum of the true negatives and false positives. Analytic specificity can also be expressed in terms of the analytic false positive rate. The analytic false positive rate is the proportion of positive test results when no detectable mutations are present (1-analytic specificity). False positive results could be due to technical errors in the analytic phase (e.g., errors in placement, contamination, expired reagents, or non-specific reactions) or to administrative/clerical errors in the pre-analytic or post-analytic phases (e.g., mislabeling of samples, wrong interpretation of correct results, or copying results incorrectly).

Wrong mutations are a third type of error, along with false negative and false positive results. These occur when a mutation is present, but is incorrectly identified (e.g. base pairs are miscounted, resulting in an incorrect location of the mutation). For purposes of this review, wrong mutations will be considered false positive results, since there is an opportunity for correcting them by confirmatory testing. Wrong mutations occurring in any of the testing phases are included in the following analyses of analytic validity.

Gap in Knowledge: How should the finding of a wrong mutation influence computation of the analytic performance? The relationship between the third type of error (wrong mutation) and analytic performance has not yet been formally addressed. In this document, a wrong mutation will be considered an incorrect result, since this type of error could cause harm. For example, determination of breast and ovarian cancer risk could be affected by an incorrect mutation report. Also, family members would not receive correct information. Further, a wrong mutation finding will be treated as a false positive, since confirmatory testing of positive results will provide the opportunity to correct this type of error.

An Optimal Dataset for Analytic Validation

Few data sources exist for estimating analytic validity. Published reports of method comparisons and screening experiences provide limited information. The "true" genotypes of the tested samples are often undocumented (i.e., not confirmed by another methodology or laboratory consensus. Future analyses should be aimed at providing reliable, method-specific analytic performance estimates. One approach for collecting such data might include the following steps:

- An independent body (such as the College of American Pathologists, American College of Medical Genetics, Food and Drug Administration or the Coriell Institute of Medical Research (Camden, NJ)) would develop a standard set of samples, most of which would be randomly selected from the general population. Correct genotypes would be arrived at by consensus. Included in the standard set would be additional samples with known mutations or variants.
- The sample set would then be available for method validation. The current validation practice of having a laboratory (or manufacturer) run a series of samples with unknown genotype is inadequate, since there is no 'gold standard' with which to compare. For example, how would a laboratory running an unknown sample determine whether a positive finding is a true, or a false, positive?

Appropriate sample size for determining analytic sensitivity and specificity has been discussed in detail in an earlier ACCE report (Prenatal Cystic Fibrosis Screening via Carrier Testing – Question 11 and 12). In brief, a target sensitivity (or specificity) can be chosen, along with an

acceptable lower limit (assumed to be the lower limit of the 95% confidence interval). Given these targets, the number of necessary samples can be derived. For example, if a laboratory chose a target specificity of 98% and wanted to rule out a specificity of 90%, it would need to correctly identify at least 49 of 50 known negative samples (estimated using the binomial distribution). When the estimates approach 100% and relatively tight confidence intervals are sought, such as might be the case for *BRCA1/2* mutation testing, a single laboratory would need to invest considerable effort. All of these suggested analyses could be done using a 2x2 table, and all rates could be accompanied by 95% confidence intervals (CI).

Analytic Performance of Sequencing Tests for *BRCA1/2* Mutations

Myriad Genetic Laboratories Due to patent restrictions, full gene sequencing for clinical purposes can only be done in one laboratory in the United States. For this reason, data and methods used to calculate laboratory-specific analytic sensitivity and specificity reside only there. Publicly available data are limited to the "Technical Specifications" listed on Myriad's website (http://www.myriadtests.com/provider/doc/tech_specs_brac.pdf). A further public source of these data is a published study (Shattuck-Eidens *et al.*, 1997) that states "sensitivity of the sequence analysis was at least 98 percent in validation studies using blinded analysis of known positive controls". However, no data are provided to support this statement. According to Myriad's website:

- Analytic sensitivity of over 99 percent Myriad reports that "failure to detect a genetic variant or mutation in the analyzed DNA regions may result from errors in specimen handling and tracking, amplification and sequencing reactions, or computer-assisted analysis and data review. The rate of such errors is estimated from validation studies to be less than one percent (<1%)" According to Myriad, "In the first BRACAnalysis validation study, a total of 55 samples were used to determine analytic sensitivity. The genetic variations identified in these sample sets were previously characterized using one of the following three methods: allele specific oligonucleotide hybridization, linkage analysis or radioactive sequencing. Fifty-four of 55 samples with known mutations were analyzed with one false negative being reported. In conducting an investigation into the false negative, it was determined that there was an insufficient quantity of DNA for the sample. As a result of this finding, procedural changes were made in order to prevent recurrence of this failure mode. In particular, the volume of DNA in a microplate is now tracked via the LIMS and adjusted accordingly in order to account for evaporation and processing of the plate whenever a pre-defined DNA aliquot is removed. In addition, each specimen that tests positive for either a mutation or uncertain variant is reprocessed in order to confirm presence of the mutation or variant and during initial DNA extraction from whole blood, a second plate identical to the first is stored as a backup. In the event that the first DNA sample is consumed, the backup plate, that is identical to the first, is retrieved in order to resume processing. In practice, the current frequency of BRACAnalysis samples with insufficient quantities for DNA reprocessing was calculated to be 0.1 percent. Through the processes of well volume tracking and confirmation of all mutations and uncertain variants, we have minimized the potential impact of insufficient DNA quantities causing potential false negative results in BRACAnalysis." From these data, we estimate the analytic sensitivity to be 98.2 percent (54/55) with a 95 percent CI from 90.3 to 99.9 percent.

- Analytic specificity of over 99 percent Myriad reports that "the incidence of a false report of a genetic variant or mutation resulting from technical error is considered negligible because of independent confirmation of all genetic variants. The incidence of a false report of a genetic variant or mutation resulting from errors in specimen handling and tracking is estimated from validation studies to be less than one percent (<1%)". Myriad also reported that "the analytic specificity of BRACAnalysis was demonstrated to be 100% (46 of 46 samples with no known mutation were analyzed with no false positives reported)." From these data, we estimate the analytic specificity to be 100 percent (95 percent CI 92.3 to 100%)."

- BRACAnalysis Large Rearrangements Analytic Validity The BRACAnalysis Large Rearrangements was designed to detect five specific large rearrangements, either deletions or duplications, in the BRCA1 gene. Positive samples for this assay were obtained from a variety of researchers and clinicians. Internal samples obtained from Myriad Genetics, Inc. (the research section of Myriad) were also used.

  According to Myriad, "analytic validity of the assay was determined with a total of 27 samples, composed of 10 samples with known large rearrangements and 17 samples with no known large rearrangements. The analytic specificity for BRACAnalysis Large Rearrangements was determined to be 100.0% (17 of 17 samples with no known large rearrangements were analyzed with no false positives reported), while the analytic sensitivity was determined to be 100.0% too (10 of 10 samples with known large rearrangements were analyzed with no false negatives reported)." From these data we estimate the analytic sensitivity to be 100 percent (95% CI 69.2-100). The analytic specificity was calculated to be 100 percent (95% CI 80.5-100).

  Gap in Knowledge: Are the data from which Myriad Genetic Laboratories estimates analytic sensitivity and analytic specificity sufficient? Myriad has provided limited data used to estimate the analytic performance of its sequencing technology. Estimates of analytic sensitivity and specificity for this laboratory cannot be considered robust. There is no appropriate external proficiency testing scheme available for blinded assessment of BRCA1/2 sequencing and it is unlikely that one will become available in the future. Testing is limited, in this instance, to one laboratory using a 'home brew' technology. In this unusual situation, new creative approaches to establishing analytic performance estimates need to be developed. In the absence of these new approaches, it is unlikely that better estimates of analytic performance will be forthcoming.

Analytic Performance of Multiple Methodologies for *BRCA1/2* Mutations

External Proficiency Testing in Europe: The European Molecular Genetics Quality Network (EMQN). The EMQN (www.emqn.org) was established in 1997 as an independent organization to provide External Quality Assessments (EQA) of molecular genetic tests. EMQN also promotes internal quality assurance by funding meetings to discuss "best practice" in disease and non-disease specific areas. The EQA schemes for the molecular diagnosis of familial breast/ovarian cancer gene mutations (*BRCA1/2*) were presented from 1999 to 2002 to assess the sensitivity of screening for unknown mutations in specified exons. Nineteen countries were represented through 2002. All laboratories used an automated DNA sequencing methodology to

identify mutations for these challenges. However, a variety of screening methodologies was used to scan the specified exons. Table 2-1 shows the results of these studies, and Table 2-2 provides the calculation of the analytic sensitivity for the participating laboratories. The overall error rate for 1999 to 2002 is 2.7 percent (95 percent CI 1.6 to 4.2%). Incorrect responses included those that identified the sequence change, but either described the mutation with incorrect nomenclature or did not include a biological interpretation (i.e. the effect of the gene mutation on the protein function). No laboratory failed all challenges. Laboratories participating in these schemes include independent diagnostic facilities, parts of genetic/oncology centers, and research institutions.

Table 2-1. *BRCA1/2* Mutation Testing: Results of the European Molecular Genetics Quality Network Survey

Year	Number Of Labs	Alleles Tested	Result		Type of Incorrect Result		
			Correct N (%)	Incorrect N (%)	False Positive N (%)	False Negative N (%)	Wrong Mutation N (%)
1999*	14	80	78 (97.5)	2 (2.4)	1 (1.2)	1 (1.2)	0 (0.0)
2000	24	136	132 (97.1)	4 (2.9)	0 (0.0)	3 (2.2)	1 (0.7)
2001*	41	238	230 (96.6)	8 (3.4)	0 (0.0)	5 (2.1)	3 (1.3)
2002*	37	216	212 (98.1)	4 (1.9)	0 (0.0)	4 (1.9)	0 (0.0)
All	116	670	652 (97.3)	18 (2.7)	1 (0.15)	13 (1.9)	4 (0.6)

* Contained *BRCA1* mutations only

Table 2-2. Analytic Sensitivity for Identifying *BRCA1/2* Mutations According to Data from the European Molecular Genetics Quality Network Survey

Year	Analytic Sensitivity (%)	(95% CI)
1999	98.8	(93.2-99.9)
2000	97.1	(92.6-99.2)
2001	96.6	(93.5-98.5)
2002	98.2	(95.3-99.5)
All	97.5	(96.0-98.5)

In addition to genotyping, the EMQN scheme also attempted to score interpretation of results. It is EMQN's position that reports should contain all relevant data to make the report a 'stand alone' source of information pertaining to the case in question. The criteria for scoring include:

- Are the patient's personal data (e.g., name, date of birth) clearly given?
- Has the clinical context been restated or has the clinical question been repeated?
- Are the results clearly presented?
- Has a clinical genetic interpretation of the results been given?
- With a negative result, have the limits of the applied test been mentioned?
- Have further options (for genetic testing and/or clinical management) been suggested?

The maximum interpretation score for each case is 2.0. The sum of the three cases is divided by three to compute the laboratory's score. The mean interpretation scores for 1999 through 2002 were 1.61, 1.38, 1.51, and 1.77. Frequent reasons for deducting points were:

- not mentioning further diagnostic options suitable to improve and/or complement the present test
- not mentioning the limits of the tests done
- incomplete interpretation of the consequences of the observed base pair change
- not mentioning that the result for a specific case increases the woman's cancer risk

Gap in Knowledge: EMQN: Analytic performance estimates are limited to sensitivity. While these data are not complete or robust, there appears to be no evidence of a problem in detecting a variety of *BRCA1* and *BRCA2* mutations, including variants of uncertain clinical significance, with any of the existing laboratory methodologies. Expansion of these challenges to include samples without mutations will help to provide estimates of analytic specificity.

Gap in Knowledge: EMQN: Analytic performance estimates are limited by the fact that laboratories are told which exons to examine. The DNA analysis of the *BRCA1/2* genes is time-consuming and expensive. However, by instructing the participating laboratories to examine only one to three exons in these challenges, the EMQN falls short of assessing actual analytic performance in most clinical or research settings.

Analytic Performance of Single and Multi-site Tests in the United States

The ACMG/CAP Molecular Genetics Laboratory Survey Ten laboratories other than Myriad provide clinical DNA testing for the three common Ashkenazi Jewish *BRCA1/2* mutations, as well as single-site testing for specific mutations known to exist in given families (www.genetests.org). The American College of Medical Genetics/College of American Pathologists (ACMG/CAP) Molecular Genetics Laboratory Survey External Proficiency Testing Program provides challenges for these laboratories. Few other data sources exist for estimating analytic validity in the United States. Published reports of method comparisons use direct sequencing as the "gold standard", assuming that it has the highest accuracy. The ACMG/CAP Molecular Genetics Laboratory Survey provides a source of data that has several advantages, including: a large proportion of clinical testing laboratories that represent the range of methodologies presently being used and samples for distribution that have

confirmed genotypes. However, basing analytic performance estimates on external proficiency testing also has drawbacks, including:

- the distributions are limited to the three *BRCA1/2* mutations common in Ashkenazi Jewish individuals
- some laboratories participating in the scheme may be from outside the United States
- the sample preparation is artificial, as is shipping and handling to ensure stability
- some participating laboratories are involved with research, rather than clinical, activities

As part of ACMG/CAP external proficiency testing in the United States, purified DNA from established cell lines derived from human cells with known mutations (http://locus.umdnj.edu/ccr/) is distributed to enrolled laboratories. The first challenge in the area of familial cancer testing, *BRCA1/2* mutation testing, was presented to 17 laboratories in 2001 and 11 laboratories in 2002. This scheme was limited to three predominant Ashkenazi Jewish *BRCA1/2* mutations, since laboratories in the U.S. are licensed only to perform this testing. *BRCA1/2* mutation challenges were all heterozygous. Table 2-3 shows the number of alleles tested and the results from the ACMG/CAP Molecular Genetics Laboratory (MGL) Survey in 2001 and 2002. All laboratories correctly classified all three specimens in both years, resulting in a 0 percent error rate, 100 percent analytic sensitivity, and 100 percent analytic specificity (Table 2-4). Appendix A contains a complete listing of the sample challenges, the responses along with the type of error (e.g., false positive), and any other adjustments made during the analysis (e.g., laboratory did not test the second allele when a mutation was identified in the first). In addition to being the first challenge by ACMG/CAP in the area of familial cancer testing, this is also the first disease target that might be approached by a DNA sequencing assay. In fact, 45 percent of the participating laboratories utilized a manual or automated sequencing approach, 24 percent used allele-specific oligonucleotide probe hybridization, and the remainder relied on some combination of allele-specific amplification and/or restriction endonuclease digestion.

Table 2-3. *BRCA1/2* Mutation Testing: Results of the ACMG/CAP Molecular Genetics Laboratory Survey

Year	Number Of Labs	Alleles Tested	Correct N (%)	Incorrect N (%)
2001	17	77	77 (100)	0 (0)
2002	11	66	66 (100)	0 (0)
All	28	143	143 (100)	0 (0)

Table 2-4. Analytic Performance for Identifying *BRCA1/2* Mutations According to Data from the ACMG/CAP Molecular Genetics Laboratory Survey

Year	Analytic Sensitivity (%)	(95% CI)	Analytic Specificity (%)	(95% CI)
2001	100	(88.1-100)	100	(92.6-100)
2002	100	(84.6-100)	100	(92.0-100)
All	100	(93.0-100)	100	(96.0-100)

Complicating factors in interpreting survey results These were limited challenges, and in each of the two years, two of the three samples did not contain a mutation. An additional aim of these external challenges was to assess the type of reporting and counseling information that the participating laboratories might include in their report. The results for interpretation are given in Table 2-5. In 2001, for each of the two challenges without a *BRCA1/2* mutation, 10 of 17 laboratories (59%) responded that the risk of breast cancer could not be determined without testing an affected relative, while 7 (41%) stated that the risk of breast cancer is the same as that for the general population. The first interpretation is correct. Responses improved in 2002, with one exception (one laboratory correctly found no mutation in the sample, but indicated a lifetime risk of breast cancer of 50 to 85% - Table 2-5). In the challenge containing a *BRCA2* mutation, 16 of 17 laboratories (94%) estimated the lifetime risk of breast cancer as 50 to 85 percent. The one remaining laboratory estimated the risk of breast cancer to be 80 to 95 percent. These post-analytic issues are as important as technical proficiency.

Gap in Knowledge: ACMG/CAP: Analytic performance estimates are preliminary. While these data are not complete or robust, there is no evidence of a problem in detecting a specific *BRCA2* mutation with any of the existing laboratory methodologies. Expansion of these challenges to include different types of mutations and comparisons amongst methodologies will assist in validating the analytic performance of the U.S. laboratories providing clinical testing for a subset of *BRCA1/2* mutations.

Gap in Knowledge: ACMG/CAP: Analytic performance estimates are available for only a small number of mutations. Only a small number of mutations (3) is included in external proficiency testing exercises (185delAG, 5382insC, 6174delT). Only one of these three mutations was challenged in the first two years. Other mutations, such as those identified in index cases, have not yet been subject to external proficiency testing.

Table 2-5. Responses for the post-analytic aspects of *BRCA1/2* mutation challenges from laboratories participating in the ACMG/CAP Molecular Genetics Laboratory Surveys

Response	Participants N (%) 2001	2002
Case 1 (no mutation)		
Lifetime risk of breast cancer is reduced but cannot be determined without *BRCA* mutation testing of an affected relative	10 (59)	10 (91)
Lifetime risk of breast cancer is the same as that in the general population	7 (41)	1 (9)
Case 2 (no mutation)		
Lifetime risk of breast cancer is reduced but cannot be determined without *BRCA* mutation testing of an affected relative	10 (59)	9 (82)
Lifetime risk of breast cancer is the same as that in the general population	7 (41)	1 (9)
Lifetime risk of breast cancer is approximately 50-85%	0	1 (9)
Case 3 (6174delT)		
Lifetime risk of breast cancer is approximately 50-85%	16 (94)	11 (100)
Lifetime risk of breast cancer is approximately 80-95%	1 (6)	0

Appendix A. Data Used to Estimate Analytic Sensitivity and Specificity from external proficiency testing

European Molecular Genetics Quality Network Table 2-6 summarizes the familial cancer testing (*BRCA1/2* mutations) external proficiency testing results obtained by European Molecular Genetics Quality Network (EMQN). Samples with known genotypes were distributed to participants from 1999 through 2002. The first column of the table contains the case number for the year. The second column contains number of participating laboratories, followed by the genotype of the sample. The number of laboratories reporting specific genotypes is then provided, along with a tabulation of their 'correct' and 'incorrect' responses. The table also contains the data used to compute the analytic sensitivity and specificity in a box, along with the yearly (and summary) totals.

Table 2-6. Computations for the EMQN Proficiency Testing Surveys

			Reported Alleles	
Distribution	**Labs**	**Genotype**	**Correct**	**Incorrect**
1999				
Case 1	**13**	**C140T**		
	12	C140T	24	0
	1	Wild type	1	1 (fn)
Case 2	**14**	**A5176G**		
	14	A5176G	28	0
	1	*		1 (fp)
Case 3	**13**	**C4446T**		
	13	C4446T	26	0
Totals	**80 alleles**		**79**	**2**
Sensitivity		24+1+28+26/80		

* One laboratory identified the correct mutation, but also reported a second base exchange that was not seen by the 2 reference labs or any of the other participants.

fn = false negative
fp = false positive

(Table 2-6 continued on next page)

Table 2-6 (Continued)

Distribution	Labs	Genotype	Reported Alleles: Correct	Reported Alleles: Incorrect
2000				
Case 1	**24**	**185delAG**		
	24	185delAG	48	0
Case 2	**24**	**1259delG**		
	22	1259delG	44	0
	1	Wild type	1	1 (fn)
	1	Wrong position	1	0 (wm)
Case 3	**20**	**A10462G**		
	18	A10462G	36	0
	2	Wild type	2	2 (fn)
Totals		**136 alleles**	**132**	**3**
Sensitivity		48+44+1+1+36+2/136		

fn = false negative, wm = wrong mutation

Distribution	Labs	Genotype	Reported Alleles: Correct	Reported Alleles: Incorrect
2001				
Case 1	**41**	**3600del11**		
	39	3600del11	78	0
	1	Wild type	1	1 (fn)
	1	4600del11	1	1 (wm)
Case 2	**38**	**G4603A**		
	34	G4603A	68	0
	2	Wild type	2	2 (fn)
	2	G4603T	2	2 (wm)
Case 3	**40**	**G5075A**		
	38	G5075A	76	0
	2	Wild type	2	2 (fn)
Totals		**238 alleles**	**230**	**8**
Sensitivity		78+1+1+68+2+2+76+2/238		

fn = false negative, wm = wrong mutation

(Table 2-6 continued on next page)

Table 2-6 (Continued)

Distribution	Labs	Genotype	Reported Alleles	
			Correct	Incorrect
2002				
Case 1	**36**	**5677insA**		
	34	5677insA	68	0
	2	Wild type	2	2 (fn)
Case 2	**36**	**300T>G**		
	36	300T>G	72	0
Case 3	**36**	**3875del4**		
	34	3875del4	68	0
	2	Wild type	2	2 (fn)
Totals		**216 alleles**	**212**	**4**
Sensitivity		68+2+72+68+2/216		

fn = false negative

American College of Medical Genetics/College of American Pathologists Table 2-7 summarizes the familial cancer testing (*BRCA1/2* mutations) external proficiency testing results obtained by the American College of Medical Genetics and the College of American Pathologists (ACMG/CAP). Samples with known genotypes were distributed to participants in 2001 and 2002. The first column of the table contains the distribution label (e.g. MGL-07 indicates the 7th DNA sample distributed as part of the Molecular Genetics Laboratory Survey). The second column contains the number of participating laboratories, followed by the genotype of the sample. The number of laboratories reporting specific genotypes is then provided, along with a tabulation of their 'correct' and 'incorrect' responses. The table also contains the data used to compute the analytic sensitivity and specificity in a box, along with the yearly (and summary) totals.

Table 2-7. Computations for the ACMG/CAP Proficiency Testing Surveys

			Reported Alleles	
Distribution	**Labs**	**Genotype**	**Correct**	**Incorrect**
2001				
MGL-07	**12**	**normal**		
	12	normal	24	0
MGL-08	**12**	**normal**		
	12	normal	24	0
MGL-09	**17**	**6174delT**		
	17	6174 delT	29*	0
Totals	**77 alleles**		**77**	**0**
Sensitivity			29/29	
Specificity			(24 + 24)/(24 + 24)	

* Five laboratories did not test the second allele when the mutation was identified in the first allele.

(Table 2-7 continued on next page)

Table 2-7 (Continued)

Distribution	Labs	Genotype	Reported Alleles	
			Correct	Incorrect
2002				
MGL3-04	**11**	**normal**		
	11	normal	22	0
MGL3-05	**11**	**normal**		
	11	normal	22	0
MGL3-06	**11**	**6174delT**		
	11	6174 delT	22	0
Totals	**77 alleles**		**77**	**0**
Sensitivity			22/22	
Specificity			(22 + 22)/(22 + 22)	

Appendix B. Analytic Methodologies Used for *BRCA1/2* Mutation Analysis

Testing Methods by U.S. Laboratories Table 2-8 lists categories of methodologies that are used to detect *BRCA1/2* mutations by laboratories participating in proficiency testing programs in the United States (ACMG/CAP MGL Survey), along with the proportions using each method. Because many laboratories utilize "home brew" assays, these categories are not homogeneous.

Table 2-8. Testing Methods Utilized by US Laboratories, According to ACMG/CAP External Surveys

Testing Method	2001 (%)	2002 (%)
Total Number of Laboratories	17	11
Allele Specific Oligonucleotide (ASO)	23.5	25.0
DNA sequencing, automated	11.8	8.3
DNA sequencing, manual	5.9	0
DNA sequencing, automated & Allele-specific PCR/ARMS	5.9	0
Allele-specific PCR/ARMS	5.9	25.0
Restriction endonuclease digestion and electrophoresis for size analysis	5.9	0
Restriction endonuclease digestion and electrophoresis for size analysis & DNA sequencing, automated	5.9	25.0
Restriction endonuclease digestion and electrophoresis for size analysis & Allele-specific PCR/ARMS	5.9	0
Restriction endonuclease digestion and electrophoresis for size analysis & Mutation Scanning Methods (SSCP, dHPLC, etc.)	5.9	8.3
Other & DNA sequencing, automated	5.9	0
Other & Restriction endonuclease digestion and electrophoresis for size analysis	5.9	0
Other & Oligonucleotide ligation assay & Restriction endonuclease digestion and electrophoresis for size analysis & DNA sequencing, automated & Allele specific PCR/ARMS & Mutation scanning	5.9	0
Other	5.9	8.3

Testing Methods in the European Community Laboratories participating in the European Molecular Genetics Quality Network (EMQN) external proficiency testing schemes from 2000 through 2002 used a variety of methods to screen for mutations. Of the 296 samples analyzed during these years, the following methodologies were used: denaturing high performance liquid chromatography (73), denaturing gradient gel electrophoresis (40), protein truncation test (39). For 144 additional samples, either no details about methodology were given, or individual exotic techniques were used.

ANALYTIC VALIDITY

Question 11. Is an internal quality control (QC) program defined and externally monitored?

Summary

- Internal quality control procedures are well described in governmental and professional published standards and guidelines
- External monitoring is provided through inspections conducted by accrediting organizations such as the Clinical Laboratory Improvement Amendments (CLIA), the College of American Pathologists (CAP) and New York State
- Myriad Genetic Laboratories is certified by both CLIA and New York State

Definition

Internal quality control is a set of laboratory procedures designed to ensure that the test method is working properly. An internal quality control program includes documentation that high standards are being practiced to ensure that:

- reagents used in all aspects of genetic testing are of high quality to allow successful test completion,
- all equipment is properly calibrated and maintained,
- good laboratory practices are being applied at every level of genetic testing. To the extent possible, all steps of the testing process must be controlled.

Quality control procedures

Techniques that are used for analyzing DNA in testing for predisposition to breast/ovarian cancer are the same as those used for other molecular testing. These techniques are widely applied and well understood. As a result, it has been possible to design and publish generic internal quality control procedures, which many molecular laboratories already have in place. Table 2-9 lists published guidelines that, among other topics, describe reagent quality control, equipment calibration and maintenance, education of the technical staff, and other internal quality control procedures. The purpose of the quality control procedures is to rigorously control all steps of the DNA testing process to minimize the potential for test failure. Given that the internal procedures for establishing and maintaining good laboratory practice are readily available (Neumaier *et al.*, 1998), the important next step will be to encourage, assist, and require laboratories to apply and document appropriate quality control procedures.

Table 2-9. Guidelines, Recommendations, and Checklists that Address Internal Quality Control Issues and Requirements.

Guidelines, Recommendations and Checklists	Source / Reference
Clinical Laboratory Improvement Amendments of 1988	Federal Register 1992;57:7002-3
Genetic Testing Under CLIA	Federal Register 2000;65: 25928-24934
New York State Laboratory Standards (9/00)	www.wadsworth.org/labcert/download.htm
Molecular Diagnostic Methods for Genetic Diseases: Approved Guidelines	National Committee for Clinical Laboratory Standards MM1-A Vol 20 #7
College of American Pathologists Checklist	www.cap.org
Standards and Guidelines for Clinical Genetics Testing	American College of Medical Genetics www.faseb.org/genetics/acmg/stds

External monitoring

All clinical laboratories performing genetic testing must comply with general regulations under the Clinical Laboratory Improvement Amendments (CLIA), and a CLIA certification should be considered the minimum acceptable level of external monitoring. One shortcoming of having only a CLIA certification is that CLIA inspectors often have less experience in evaluating genetic testing laboratories than other certifying organizations. CLIA is in the process of upgrading its regulations regarding genetic testing. The Task Force on Genetic Testing concluded that the current CLIA requirements are insufficient to ensure quality of molecular genetic testing. Laboratories certified by the College of American Pathologists (CAP) or by New York State Health Department will have undergone a more rigorous external monitoring that requires specific procedures and documentation. Myriad Genetic Laboratories is currently CLIA-certified and is licensed by New York State.

ANALYTIC VALIDITY

Question 12. Have repeated measurements been made on specimens?

Summary

- Having information about repeated measurements on the same specimen is important for determining the type and rate of errors in *BRCA1/2* mutation testing
- External proficiency testing programs in the U.S. provide limited data for repeated measurements on the same specimen by multiple laboratories.
- To date, all participating laboratories (including Myriad Genetic Laboratories) have agreed on the mutation status of all challenges
- All clinical laboratories test control samples repeatedly, but results are not usually reported
- Myriad Genetic Laboratories has performed internal method comparisons showing a high degree of agreement between gel-based and capillary-based sequencing

Measurements made on the same specimen in different laboratories

Multiple laboratories have made repeated measurements on the same specimen, utilizing a variety of technologies. A collaborative external proficiency testing program, jointly administered by the American College of Medical Genetics and the College of American Pathologists (ACMG/CAP) provided three breast/ovarian cancer predisposition DNA challenges in 2001 and another three in 2002. A summary report of the results was also provided (Questions 9 and 10 give more detail). In the first two distributions of *BRCA1/2* mutations, 28 of 28 participating laboratories (100%, 95 percent CI 93.0-100%) correctly reported the results for all three challenges. This survey was limited to the three predominant Ashkenazi Jewish *BRCA1/2* mutations, and only one of the three samples in each year contained a mutation. The European Molecular Genetics Quality Network (EMQN) schemes for the molecular diagnosis of familial breast/ovarian cancer gene mutations (*BRCA1/2*) were presented from 1999 to 2002 (full data are not yet available from 2002) to assess the sensitivity of screening for unknown mutations in specified exons. Forty-one laboratories from eighteen countries were represented through 2001. All laboratories used an automated DNA sequencing methodology to identify mutations for these challenges. However, a variety of screening methodologies was used to scan the specified exons. The overall error rate for 1999 to 2002 is 2.7 percent (95 percent CI 1.6 to 4.2%). The overall sensitivity is 97.1 percent (95% CI 95.2-98.5 percent - Questions 9 and 10).

Measurements made repeatedly on the same sample within a laboratory

It is common practice for repeated measurements to be made on the same specimen (a control specimen) within a laboratory. For each assay, a positive control is usually included for testing. This internal documentation will remain within the laboratory but will be available for on-site inspections by certifying agencies as part of external monitoring. Thus, one avenue for collection of these data would be to use laboratory survey instruments. For DNA sequencing at Myriad Genetic Laboratories, typical controls (positive or negative) are not run. Clinical Laboratory Improvement Amendments (CLIA) has approved a quality control protocol that involves the correct sequencing from each batch of reagents, independent analysis of all deleterious and uncertain mutations, assurance of no contamination, and control of the majority

of the sequence against 15 other specimens (16 specimens per run - Ward B, personal communication).

Myriad Genetic Laboratories has described an annual internal proficiency testing program, where 12 samples containing a wide range of known deleterious *BRCA1/2* mutations are sent to clients who then blindly resubmit them (Ward B, personal communication). Since January 1997, a total of 144 such internal proficiency tests have been conducted for BRACAnalysis. The proficiency tests were conducted using a set of DNA samples with known mutations, and no sample was sent more than once. These samples represented more than 750 recurring genetic variations in the *BRCA1/2* genes. To date, all 144 proficiency tests have identified the same genetic variants as the initial test. In addition, whenever there is a major change to its automated sequencing methodology, Myriad performs a revalidation using 80 samples with known deleterious *BRCA1/2* mutations. This has occurred approximately once a year since 1998. Data from these exercises are not available for our analysis.

According to Myriad, "A number of internal validation studies have been conducted in order to ascertain the analytic validity of BRACAnalysis. Most recently, a large scale study comparing data between gel-based and capillary-based sequencers was conducted with internal samples. Samples were first characterized using gel-based sequencing in order to identify genetic variations in the BRCA1/2 genes. Following gel-based sequencing, the same sample set was analyzed using capillary-based sequencing." Among the 128 samples with a mutation identified by the gel-based methodology, the capillary-based methodology agreed in all instances. Among the 910 samples without a known mutation, the two methods also agreed in all instances.

ANALYTIC VALIDITY

Question 13. What is the within- and between-laboratory precision?

This question is not applicable to testing for predisposition to breast/ovarian cancer, since such testing is qualitative. This question is only relevant to quantitative measurements such as repeat sizing.

ANALYTIC VALIDITY

Question 14. If appropriate, how is confirmatory testing performed to resolve false positive results in a timely manner?

Summary
- Confirmatory testing is additional testing to verify the finding of a mutation(s)
- It is likely to be useful because of occasional false positive test results
- There is little information about how often confirmatory testing corrects an error

Definitions

Confirmatory testing is performed to ensure that the initially positive test result is correct. Examples include:

- a *BRCA1/2* mutation, either known deleterious or variant of uncertain significance, is identified in an individual. The specimen is then re-run to ensure that the result is correct.
- a methodology other than sequencing (e.g. protein truncation test) suggests a mutation. Sequencing is then used to identify and describe the mutation.

Four distinct types of confirmatory testing could be utilized, depending on the testing protocols in place and the circumstances in which the positive test result is obtained.

- Repeating the same test protocol on another aliquot of the same specimen
- Repeating the same test protocol on a different specimen
- Performing a different test protocol on another aliquot of the same specimen
- Performing a different test protocol on a different specimen

Reflexive testing is different from confirmatory testing, in that if a single or multi-site analysis does not identify a *BRCA1/2* mutation, full sequencing can be performed.

Importance of confirmatory testing

It is important to determine how often 'false positive' results will be identified upon confirmatory testing. Based on the European proficiency testing experience, false positive results may occasionally occur (Question 9, Table 2-1). For this reason, it may be useful to perform confirmatory testing, when a mutation is identified. This issue is dealt with in more detail under Clinical Validity (Questions 19 and 20). Myriad Genetic Laboratories routinely confirms all positive test results by repeating the same test protocol on another aliquot of the same specimen.

Gap in Knowledge: Impact of confirmatory testing on analytic specificity. Myriad Genetic Laboratories routinely performs confirmatory testing on all positive test results, but information is not currently publicly available to determine the impact of confirmatory testing on analytic sensitivity and, consequently, the overall screening process.

ANALYTIC VALIDITY

Question 15. What range of patient specimens has been tested?

Summary

- Whole blood, fresh or frozen tissue and buccal samples are acceptable for *BRCA1/2* mutation testing for all types of DNA analysis
- Paraffin-embedded tissue is only suitable for single- and multi-site DNA analysis only

Molecular genetic *BRCA1/2* mutation analysis has been successfully performed in a variety of specimens using available methodologies. DNA testing can be performed on:

- whole blood (DNA isolated from peripheral blood lymphocytes)
- fresh or frozen tissue
- paraffin embedded tissue samples from tumors (single- and multi-site analysis only)
- buccal samples

ANALYTIC VALIDITY

Question 16. How often does the test fail to give a useable result?

Summary

- Laboratory testing for *BRCA1/2* mutations can be divided into pre-analytic, analytic and post-analytic phases
- In the pre-analytic phase, generally agreed upon criteria are in use to determine the appropriateness of testing. If these are not met, the test can be canceled
- In the analytic phase, samples fail for multiple reasons, and these failures are routinely documented in clinical laboratories but are not generally available for outside review
- When analytic failures do occur, repeating the analysis will often yield useable results
- Types of failures and their associated rates are rarely reported

Test 'failures' in the pre-analytic phase of testing

In the pre-analytic phase, it may be determined that the sample is not suitable for testing because specific clinical criteria are not met, or because the sample is considered inadequate. While programs often monitor pre-analytic test cancellation rates as part of an overall quality assurance plan, these events are usually not considered a laboratory or methodologic 'failure'. Table 2-10 lists criteria commonly used for deciding whether to reject a sample in the pre-analytic phase.

Table 2-10. Common Pre-analytic Criteria for Rejecting a Sample Submitted for *BRCA1/2* Mutation Testing

Rejection Criteria Based on Clinical Information
Inability to demonstrate informed consent
Inappropriate referral (e.g. a genetic counselor referral from a state where counselors are not authorized to refer)
Rejection Criteria Based on Submitted Sample
Inadequate specimen quality (e.g., hemolyzed blood or obvious contamination)
Inappropriate sample (e.g., whole blood with no anticoagulant or wrong anticoagulant)
Inadequate specimen labeling
Inappropriate handling prior to laboratory receipt (e.g., sample too long in transit or exposed to extreme temperature)

Test failures during the analytic phase of testing

Failures of individual samples or assays occur when preset quality control standards are not met and, therefore, test results are not considered reportable. Failures can arise for a number of reasons, such as improperly processed samples, problems with component reagents, or equipment malfunction. Many assay failures within the clinical molecular genetic laboratory are due to operator error. Automation and programs to properly train laboratory personnel can help

avoid these problems. Only a few medical technology programs, however, currently provide adequate molecular components. Documentation of failures and subsequent corrective action is required by regulatory agencies such as CLIA and the College of American Pathologists. Unfortunately, failure rates and other information on assay robustness are often not published. Available data suggest, however, that repeating the analysis of an individual sample or assay run can often yield a satisfactory result. An estimated 10 percent of amplicons (*BRCA1* is divided into 36 amplicons and *BRCA2* into 48 amplicons) undergoing DNA sequencing at Myriad Genetic Laboratories are re-analyzed, due to unacceptable quality of data/amplifications or for mutation confirmation (Ward B, personal communication).

An irretrievable assay failure occurs when an apparently suitable specimen is submitted and approved for testing, but the assay yields a result that is clinically uninterpretable. Failures of this type are most often related to the quality of the original sample. Procedural problems during specimen processing and DNA extraction can also be responsible. Success rates for obtaining clinically interpretable results are close to 100% for blood samples.

Post-analytic failures, such as incorrectly or inadequately interpreted results, are considered separately from analytic test failures, as part of a review of overall quality assurance in the Clinical Utility section (Question 32).

Gap in Knowledge: Overall, and method-specific, failure rates. Clinical laboratories are required to document test failures. Test failure rates could be provided by laboratories participating in external proficiency testing administered by ACMG/CAP. Myriad Genetic Laboratories has provided the proportion of tests that fail initially, but has not yet provided information about how often the repeated analysis is successful.

ANALYTIC VALIDITY

Question 17. How similar are results obtained in multiple laboratories using the same, or different, technology?

Summary

- Data derived from external proficiency testing can be used to judge the consistency of results from laboratories testing for *BRCA1/2* mutations
- External proficiency testing in the U.S. does not currently yield useful information for laboratories using sequencing methodology
- External proficiency testing in Europe does not currently yield useful information for laboratories using sequencing methodology

Comparing results from different laboratories using the same or similar methodologies

One potential source of data for evaluating differences in *BRCA1/2* mutation test results from multiple laboratories using the same (or a similar) method would be external proficiency testing. However, the small number of participants in ACMG/CAP (17) and the relatively large number of methods (Table 2-8, Appendix B) preclude obtaining meaningful method-specific analyses. The number of participants in EMQN is greater (41), however, the methodologies used are not published. Even if available, such comparisons might be complicated because laboratories in the same methodological category could use different commercial or in-house reagent components and protocols. For example, although three laboratories might be grouped under the ARMS™ methodology, one might use a prepared kit, a second might use commercially prepared analyte specific reagents (ASR), and the third might use in-house reagents. These factors would make the comparison nearly equivalent to comparing different methodologies. To help in comparing methodologies, the ACMG/CAP MGL Survey Reports and EMQN EQA schemes might consider stratifying results into broad methodological categories.

Comparing results from different laboratories regardless of the methodology

As part of the 2001 ACMG/CAP Molecular Genetics Laboratory external proficiency testing survey, 17 laboratories were queried about their methodology for performing *BRCA1/2* mutation analysis (Table 2-8, Appendix B). Limited data are currently available. To date, method-specific data on error rates are not available from these surveys. However, for this challenge there was a high level of agreement between laboratories for detecting mutations that were targeted by their specific method.

The EMQN scheme reported results from a number of laboratories using surveys from 1999 to 2002. These laboratories used a variety of methods to pre-screen for *BRCA1/2* mutations (Appendix B). To date, method-specific data on error rates are not available from these surveys. However, for this challenge there was a high level of agreement between laboratories for detecting mutations that were targeted by their specific method.

> **Gap in Knowledge: Comparing results from different laboratories with the same methodology.** There are no current data that compare results from different laboratories with the same methodology for *BRCA1/2* mutation testing.

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